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Relationship between ceruloplasmin and Cu status involving metallothionein induced by several heavy metals in the mouse

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Abstract. ICR male mice aged 5 weeks were injected subcutaneously with $CdCl_2$, $Pb(CH_3COO)_2$, $AgNO_3$, $CuCl_2$, a combination of Cd and Ag compounds, or a combination of Cu and Ag compounds. These injections were carried out 3 times. Twenty-four hours after the last injection, they were sacrificed. Cd injection significantly stimulated serum ceruloplasmin (Cp) activity and Cu concentration, accompanied by an increase in hepatic Cu. Pb injection also slightly increased the Cp level. In contrast, Ag injection markedly decreased both Cp activity and Cu concentration in the serum. Hepatic Cu increased slightly after Ag injection. Using a combination of Cd and Ag, only the Ag effect on the Cp activity appeared. The Cu injection stimulated Cu binding to metallothionein (MT) and bile excretion of Cu, but not Cp release. With a Cu and Ag combination, the effect of Ag on Cp was lost, with a concomitant disappearance of Ag from the Cp fraction in the serum. Our results suggest that in the mouse, Cd and Ag, Cu antagonistic metals, influence different sites of Cp metabolism. Excess hepatic Cu is partly eliminated by excretion of bile and is partly detoxified by MT induction.

Key words: Cerulaplasmin – Copper – Cadmium – Silver – Lead – Metallothionein

Introduction

A blue protein from the α2-globulin fraction of human serum first reported by Holmberg (1944) was named ceruloplasmin (Cp) by Holmberg and Laurell (1948, 1951). In 1966, Osaki et al. demonstrated that Cp possesses ferroxidase activity and oxidizes hepatic Fe (II) to Fe (III), which is then bound to transferrin. Since then, much interest has been focused on the relation between Fe metabolism and the activity of this substance (Osaki and Johnson 1969; Ragan et al. 1969; Roser et al. 1970). Now, the role of Cp as a free radical scavenger is a new object of investigation (Goldstein et al. 1979).

Cu is an essential constituent of Cp and is involved in the biosynthesis of Cp in the liver (Evans et al. 1970a). However, Cp induction by Cu administration has yielded equivocal results (Holtzman and Gaumnitz 1970; Linder et al. 1979).

Previously, we observed that acute administration of Cd and Ag, a known antagonist of Cu, evoked opposing effects on serum Cp activity (Sugawara and Sugawara 1984). However, the mechanism of action could not be clarified. We designed experiments to clarify the relationship between Cp synthesis and Cu status involving metallothionein induction, as modified by various heavy metals, including Cd and Ag.

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Materials and methods

Specific-pathogen-free male mice (ICR, 5 weeks old) were housed in groups of six to eight animals on a 12 h light-dark cycle at constant room temperature (25 ± 0.4 °C) and humidity (42 ± 5%), and free fed on a standard diet (type M from Oriental Yeast Co., LTD Tokyo Japan) containing 11 ppm Cu and tap water. Metal compounds, which were of the highest purity grade available (Wako Pure Chemical Ind., LTD Tokyo, Japan), were injected subcutaneously three times every 24 h. Single injections of Cd ($CdCl_2$), Ag ($AgNO_3$), Pb [$Pb(CH_3COO)_2$] and Cu ($CuCl_2$) were 1.5 mg Cd/kg body wt, 1.5 mg Ag/kg body wt, 20.0 mg Pb/kg body wt and 3.0 mg Cu/kg body wt, respectively. Two combination groups, Cd + Ag and Cu + Ag, were treated with Cd plus Ag, and Cu plus Ag at the same time, but at different injection sites, respectively. Only deionized water was administered to the control group.

At 24 h after the final injection, all mice were sacrificed by heart puncture. Serum was used to measure ceruloplasmin (Cp) activity and metal content. Cp oxidase activity in the serum was measured as described by Schosinsky et al. (1974). Pooled serum in the Ag and Cu + Ag groups was separated by a column (1.5 × 65 cm) packed with Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), and equilibrated with 0.1 M Tris-HCl buffer, pH 8.5, according to the method described by Sugawara et al. (1981).

A small piece of liver (about 0.5 g) was added to a mixture of deionized water (0.5 ml), nitric (1.0 ml) and perchloric (0.2 ml) acids. The sample was then boiled until the suspension became clear, was cooled at room temperature, and was passed through a funnel equipped with filter paper (type No. 2, Toyo Roshi Co., LTD., Tokyo, Japan). It was brought to a final volume of 20 ml with deionized water. This diluted solution was used for metal determination.

The remainder of the liver was homogenized with 5 volumes 0.25 M sucrose. This homogenate was centrifuged at 105000 *g* for 60 min. Centrifugation was repeated twice.

Table 1. Ceruloplasmin and metal in serum

	Cp (U/l)	Cu (μg/ml)	Cd (μg/l)	Ag (μg/ml)
Control	29.9 ± 2.4 (8)	0.81 ± 0.06 (8)	–	–
Cd	51.6 ± 10.6 (6)**	0.98 ± 0.11 (6)**	28 ± 41 (7)	
Ag	6.3 ± 4.7 (7)**	0.53 ± 0.05 (7)**	–	0.85 ± 0.09 (7)
Pb	37.6 ± 3.3 (7)**	0.87 ± 0.10 (7)	–	–
Cu	30.2 ± 10.4 (5)	0.91 ± 0.13 (6)	–	–
Cd + Ag	5.2 ± 3.1 (8)**	0.60 ± 0.02 (8)**	83 ± 102 (8)	1.21 ± 0.26 (8)
Cu + Ag	37.3 ± 8.3 (7)*	1.11 ± 0.27 (7)*	–	0.52 ± 0.42 (7)

Each value represents the mean ± SD of five to eight animals. Asterisks denote differences (by a two-tailed Student's *t*-test) between control and test groups: ** $p < 0.01$; * $p < 0.05$

The final pellet was digested with the mixture of acids as described above, and then diluted to 20 ml with deionized water. The cytosol and diluted solutions were used for the metal assays. The cytosol solution (first cytosol fraction) was also used for the determination of metal distribution by the Sephadex G-75 column technique (Sugawara and Sugawara 1984).

Bile was directly collected from the gall bladder by insertion of a capillary tube and then diluted with deionized water for Cu measurement. All metals were measured by an atomic absorption spectrophotometer equipped with a flameless graphite cuvette (Model 180-80, Hitachi Co., LTD., Tokyo, Japan) or a flame burner operated with air/acetylene (Model 208, Hitachi Co., LTD.).

Results

Ceruloplasmin activity and metal concentrations in serum

Injections of Cd and Pb significantly increased ceruloplasmin (Cp) levels (Table 1). Although the Cd dose was obout 1/13 that of the Pb dose, its effect was very marked. However, injection of Ag decreased Cp activity. The simultaneous injection of Cd and Ag (Cd + Ag group) exhibited only the action of Ag. The Cu group did not show such changes. However, when Cu was injected along with Ag (Cu + Ag group), Cp activity was somewhat higher than in controls. Cu may therefore compete with the Ag effect on Cp.

Changes in serum Cu were very similar to those of Cp in all treatment groups (Table 1). Therefore, the relationship between serum Cu concentration and Cp activity, calculated by linear regression, was significant ($r = 0.778$; $p < 0.01$).

Serum Ag levels were much higher than those of Cd (Table 1), although the doses of both metals were equal. In the three groups treated with Ag, the relation between serum Ag and Cp was significantly negative ($r = -0.788$; $p < 0.01$). There was no significant relation between Cp activity and Cd concentration. Serum Zn was low in the Cd group, but high in the other groups (data not shown). Serum Fe was not influenced by the metal injections in any of the groups (data not shown).

Distribution of Cu and Ag in serum

When serum from the Ag group was subjected to gel filtration, most of Ag (87%) was found in the void volume and the rest (13%) in the second region on the Sephadex G-200 column (Fig. 1). The second peak corresponded to gamma-globulin (Mr 160000), as shown with standard marker protein (Schwarz/Mann, NY, USA). In contrast, in the Cu + Ag group, Ag disappeared from the gamma-globulin region and was eluted in the void volume (Fig. 1). Cu was detected only in the globulin region in the two groups under our conditions. We did not assay Cp activity in this region. Quite possibly, this fraction is the Cp protein.

Cu and Fe in liver and Cu in bile

Hepatic Cu concentrations were increased to about 10 times those of control in the Cu and Cu + Ag groups (Table 2). The concentration was also increased in the Cd and Ag groups, but this increase was not statistically significant. The Cd + Ag group showed a significantly higher level of Cu than that of the control. Concentration changes

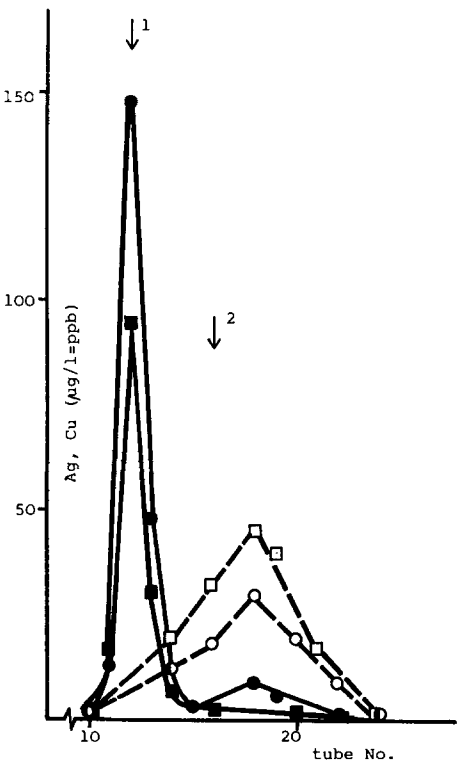

Fig. 1. Gel filtration elution profiles of Ag and Cu in serum. The column (1.5 × 65 cm) was packed with Sephadex G-200, equilibrated with 0.1 M Tris-HCl buffer, pH 8.5. The symbols represent Ag and Cu (●–● and ○–○ in the Ag group), and Ag and Cu (■–■ and □–□ in the Cu + Ag group). *Arrows 1* and *2* indicate the elution of blue dextran and gamma-globulin, respectively

Table 2. Hepatic Cu and Fe, and biliary Cu

	[a]Cu (μg/g wet)	[b]Cu (μg/g protein)	[c]Fe (mg/g protein)	Cu (μg/ml bile)
Control	4.39 ± 1.0 (8)	42.1 ± 10.9 (8)	0.68 ± 0.12 (8)	0.95 ± 0.23 (8)
Cd	5.02 ± 0.6 (7)	47.5 ± 12.3 (7)	0.75 ± 0.12 (7)	1.13 ± 0.39 (7)
Ag	5.43 ± 0.7 (7)	45.9 ± 12.1 (7)	0.68 ± 0.14 (7)	0.92,1.86 (2)
Pb	4.34 ± 0.8 (7)	41.1 ± 18.8 (6)	0.73 ± 0.07 (6)	1.07 ± 0.12 (4)
Cu	48.75 ± 8.1 (6)**	388.2 ± 74.3 (6)**	0.61 ± 0.17 (6)	9.27 ± 0.72 (3)**
Cd + Ag	6.39 ± 1.2 (8)**	42.7 ± 7.3 (8)	0.66 ± 0.13 (8)	1.04 ± 0.23 (6)
Cu + Ag	56.68 ± 12.3 (7)**	552.7 ± 62.6 (7)**	0.76 ± 0.15 (7)	9.67 ± 1.47 (5)**

Each value represents the mean ± SD
[a]Cu concentration: (μg/g wet liver). [b]Cu and [c]Fe concentrations: (μg/g protein of hepatic cytosol) and (mg/g protein of hepatic cytosol).
** $p < 0.01$ by a two-tailed Student's *t*-test

caused by Cd or Ag did not reflect the Cu concentration in the cytosol fraction (Table 2).

Hepatic Cu is mainly excreted via the bile (Owen 1974). In the Cu and Cu + Ag groups, Cu excretion was about 10 times that of the controls (Table 2). In comparison with the control group, both Cd and Pb groups showed a high, but not significant, excretion level. In the Ag group, it is not clear whether Cu excretion was increased.

Cytosolic Fe, probably ferritin-bound, was not influenced by any metal injection (Table 2).

Distribution of Cu in liver

Even in the group where hepatic Cu concentration underwent significant changes, the proportion of Cu in the cytosol fraction did not vary much (Table 3). Cu accumulated not only in the cytosol but also in other cellular fractions.

In control cytosol, 90% of the Cu was found by Sephadex G-75 column in the area between the high molecular weight (HMW) (near void volume) and metallothionein (MT) fractions (Table 3). In the MT fraction, only about 4% of the Cu was found. In the Cd and Cd + Ag groups, this portion increased to 25% of total cytosolic Cu. Ag or Pb injections (Ag or Pb groups) only slightly increased this portion (7–8%) in the MT region. In the two groups treated with Cu, the Cu and Cu + Ag groups, the portion of Cu in the MT region was over 50%. The profiles appeared to be similar to that of the rat loaded orally with Cu (Terao and Owen 1976).

Uptake of Cd and Ag into liver

Table 4 shows uptake and distribution of Cd and Ag in the liver. Hepatic Cd was recovered at a level of about 40% of the total dose, but Ag 10%. Most hepatic Cd was found in the MT fraction on Sephadex G-75. Ag in the MT fraction was 20% of the total cytosolic Ag in the Ag group. This percentage was increased to 50% and 60% by simultaneous treatment with Cu and Cd, respectively.

Discussion

As shown in Table 1, the Cu group (Cu injection alone) showed no effect on Cp activity, though its injection resulted in marked increases in hepatic Cu concentration and Cu-MT induction. When mice were given food containing 11 ppm Cu (the optimum level of Cu in mouse diet, according to the National Research Council is 4.5 ppm), excess Cu was deposited in the liver, but did not appear to contribute at all to the Cp biosynthesis (Milne et al. 1969; Brown et al. 1979). However, Evans et al. (1970b) reported that Cp concentration was significantly increased in 15-day-old and adult rats (presumably they were fed a diet with a normal Cu level) treated with Cu, but not in newborn rats.

Sugawara and Sugawara (1984) reported previously that serum Cp was stimulated by Cd injection in the mouse. Ashby et al. (1980) also demonstrated that Cd blocked the excretion of Cu in the bile, leading to an accumulation of Cu in the liver, which in turn stimulated the synthesis of Cp in the rat. We could not observe the reduction of bile Cu in the Cd-treated mice (Table 2). The increased hepatic Cu may contribute to the stimulation of Cp activity and/or biosynthesis. However, this hypothesis cannot adequately explain why Cp activity was not stimulated by excess Cu (in the Cu group) or why the stimulation effect of Cd was no longer observed in the Cd + Ag group.

Table 3. Distribution of Cu in liver

	Cyto/whole (%)	HMW (%)	MMW (%)	LMW (MT) (%)
Control	66 ± 4 (8)	6.4 ± 0.7 (3)	92.8 ± 3.8 (3)	4.1 ± 0.6 (3)
Cd	68 ± 4 (7)	5.4 ± 1.3 (3)	69.9 ± 4.3 (3)	24.7 ± 3.1 (3)
Ag	60 ± 3 (7)	11.2 ± 4.7 (3)	80.5 ± 4.4 (3)	8.3 ± 1.6 (3)
Pb	71 ± 4 (6)	6.1 ± 0.7 (3)	86.2 ± 0.8 (3)	7.6 ± 1.3 (3)
Cu	71 ± 4 (6)	29.7 ± 4.4 (3)	18.2 ± 3.4 (3)	51.9 ± 7.8 (3)
Cd + Ag	60 ± 3 (8)	9.2 ± 3.4 (3)	64.1 ± 8.3 (3)	26.7 ± 5.0 (3)
Cu + Ag	68 ± 4 (5)	18.7 ± 4.1 (3)	9.9 ± 2.2 (3)	70.8 ± 4.4 (3)

Each value represents the mean ± SD. The supernatant was separated by a Sephadex G-75 column. HMW, MMW and LMW (MT) are high, middle, and low molecular weight, respectively. The three regions were determined by our previous method (Sugawara 1977)

Table 4. Uptake and distribution of hepatic Cd and Ag

		Uptake (%)	Cytosol/whole (%)	MT/cytosol (%)
Cd	Cd	41±11 (7)	94±1 (7)	98±0 (3)
Ag	Ag	12±10 (7)	19±9 (7)	18±7 (3)
Cd+Ag	Cd	38±5 (8)	95±1 (8)	98±0 (3)
	Ag	13±4 (8)	37±10 (8)	60±5 (3)
Cu+Ag	Ag	18±12 (7)	38±7 (8)	48±7 (3)

Uptake: total hepatic metal/total dose of metal
Cytosol/whole: cytosol metal/whole hepatic metal
MT/cytosol: MT metal/cytosol metal
Cd or Ag in the MT region was estimated by a Sephadex column according to our previous method (Sugawara 1977)
Each value represents the mean ± SD

When Cd is provided orally, Cp seems to respond in a manner different from that suggested by Ashby's (1980) and our results. Whanger and Weswig (1970) indicated that serum Cp activity decreases in rats exposed orally to 25–100 ppm Cd for 8 weeks. Campbell and Mills (1974), and Sowa and Steibert (1985) also reported that rats given Cd orally showed a low level of Cp in the serum. Bremner and Campbell (1980) mentioned that the low activity of serum Cp in rats treated orally with Cd may be due to low levels of hepatic Cu due to defects in Cu absorption. We did not previously observe any significant change of serum Cp activity in mice treated orally with 200 ppm Cd for 6 weeks (Sugawara et al. 1984). These differences may depend upon the animals used or the administration route of Cd.

Stimulation by Pb of Cp was also observed (Table 1). However, its magnitude was much smaller than that of Cd (Table 1). Judging from the fact that hepatic Cu concentration was barely changed in the Pb group (Table 2), it is more probable that the effect of Pb did not occur as a result of the deposition of hepatic Cu (Klauder and Petering 1977).

In contrast, the extremely low Cp activity in the Ag group was very similar to the results earlier observed in the Cu deficient animals (Evans and Abraham 1973; Planas and Frieden 1973). However, the mice treated with Ag alone did not show a reduction in hepatic Cu (Table 2). Therefore, it is impossible to conclude that the decrease in Cp (Table 1) was due to the reduction of hepatic Cu (Table 2). In the Cd+Ag group, only an Ag effect on Cp was shown (Table 1). Our previous hypothesis (Sugawara and Sugawara 1984) that the low activity of serum Cp may be partly due to the reduction of Cu in the hepatic microsome could not be supported by this observation in the Cd+Ag group (Table 1). When Cu was given together with Ag (Cu+Ag group), excess Cu prevented the Ag effect (Table 1). This result indicates that excess hepatic Cu can compete with Ag in Cp induction.

A significant positive relationship between serum Cp and Cu suggests that serum Cu is regulated by the release of Cp from the liver, while the negative relationship between serum Cp and Ag indicates that Ag can associate with the Cp protein. If so, since Cu cannot bind to apo-Cp except in the liver, (Poulik and Weiss 1975), binding of Ag to the protein should occur in the liver. Pribyl et al. (1982) reported that the low level of serum Cp activity in the rat given Ag orally may be partly due to the Ag-Cp (Ag binding Cp) release into the blood.

Our chromatographic results (Fig. 1) and those from Cp assays (Table 1) indicate that Ag can compete with Cu at the site of apo-Cp to Cp, resulting in the release of the Ag-Cu-binding Cp to the blood. Four atoms of Cu are needed to activate the oxidase activity of Cp (Funakoshi 1983). Ag may displace one of the four Cu atoms in the active site in the Ag group. This supports our previous observation (Sugawara and Sugawara 1984) that the Ag exerts a strong effect on the Cp activity.

In conclusion, Cd or Pb injection stimulates the release of Cp into the blood, thus resulting in the enhancement of serum Cp activity. Ag incorporated into the liver competes with Cu at the apo-Cp to Cp stage and, when it associated to the Cp molecule, it brings about a consequent loss of Cp activity. The loss of this activity is presumably due to the conformational change of the Cp molecule. Although Cu is linked closely to the biosynthesis of Cp, excess Cu did not show any further release of Cp into the blood.

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